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Stability of Monacolin K and Citrinin and Biochemical Characterization of Red-Koji Vinegar during Fermentation

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ABSTRACT: Red-koji vinegar is a *Monascus*-involved and acetic acid fermentation-derived traditional product, in which the presence of monacolin K and citrinin has attracted public attention. In this study, red-koji wine was prepared as the substrate and artificially supplemented with monacolin K and citrinin and subjected to vinegar fermentation with *Acetobacter* starter. After 30 days of fermentation, 43.0 and 98.1% of the initial supplements of monacolin K and citrinin were decreased, respectively. During fermentation, acetic acid contents increased, accompanied by decreases of ethanol and lactic acid contents and pH values. The contents of free amino acids increased while the contents of other organic acids, including fumaric acid, citric acid, succinic acid, and tartaric acid, changed limitedly. Besides, increased levels of total phenolics in accordance with increased antioxidative potency, α,α-diphenyl-β-picrylhydrazyl scavenging, and xanthine oxidase inhibitory (XOI) activities were detected. It is of merit that most citrinin was eliminated and >50% of the monacolin K was retained; contents of free amino acids and total phenolics along with antioxidant and XOI activities of the red-koji vinegar were increased after fermentation.

KEYWORDS: *Monascus, red-koji vinegar, monacolin K, citrinin, organic acids, free amino acids, xanthine oxidase inhibitor*

INTRODUCTION

Red-koji, traditionally prepared by inoculation of the cooked rice with *Monascus* starter and cultivation to produce red-colored rice, is named Anka, red mold rice, or red rice in China, benikoji or akakoji in Japan, and red Chinese rice in Europe. It has been widely used as a potent source of enzymes, production of red pigment, and biosynthesis of monacolin K and other bioactive metabolites. In the preparation of some traditional Chinese foods and beverages, red-koji is applied for extensive saccharization of various grain starches in facilitation of the subsequent wine fermentation and generation of unique and pleasant flavors.[1] In addition to those traditional products, various red-koji products with some health-related bioactivities have been evaluated.[2−6]

Monacolin K is the major bioactive compound contributing to the hypocholesterolemic activity of red-koji related food products. Discovery of monacolin K bioactivity in the inhibition of cholesterol biosynthesis is a highlighted healthcare issue.[1,7−9] In particular, it has the same chemical structure as lovastatin, a commonly prescribed medicine for the treatment of hypercholesterolemia and reducing the risk of cardiovascular and cerebral vascular diseases. However, citrinin biosynthesized by *Monascus* or other contaminant molds has been identified as a toxic compound that causes kidney or liver disorders.[1,10] On toxicological consideration, elimination of citrinin is essential in the preparation of *Monascus*-related food products.

Vinegar is not only a popular ingredient in the kitchen to prepare cuisines but used anecdotally in traditional Chinese medicine as a therapeutic regimen for ameliorating clinical symptoms of gout. Its biological activities in the enhancement of metabolic rate, release of fatigue, modulation of hypertension and blood sugar levels, and prevention of cancer have been demonstrated in recent years.[11−13] Enhanced antioxidant activities have been observed in various vinegars.[13,14] Gout is the most common form of arthritis caused by hyperuricemia, a high level of uric acid in the blood. Xanthine oxidase (XO) is a critical enzyme for uric acid production, catalyzing the conversions of hypoxanthine to xanthine and xanthine to uric acid.[15] Two active compounds with xanthine oxidase inhibitory (XOI) activity have been isolated, identified, and characterized in the red-koji fermented rice vinegars.[16] In this study, an in vitro determination of XOI activity as affected by the introduction of acetic acid and red-koji vinegars was conducted.

Following the indigenous practices, red-koji wine used as substrate was inoculated with *Acetobacter* starter and incubated at ambient temperature for 30 days for red-koji vinegar fermentation. The red-koji wines were artificially supplemented with authentic monacolin K and citrinin and subjected to vinegar fermentation, and subsequent changes of their concentrations during fermentation were monitored. Furthermore, compositional changes during fermentation were analyzed and associated nutraceutical activities including antioxidative potency (AOP), α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging, and XOI activities of the red-koji vinegar were determined.

MATERIALS AND METHODS

Red-Koji Wine and Vinegar Preparation. For red-koji wine preparation, red-koji and white-koji were needed. Both were purchased from a local market. In Taiwan, *Saccharomyces cerevisiae* and *Aspergillus oryzae* are usually contained in the white-koji, and *Monascus anka* was mostly contained in the red-koji. Red-koji wine was prepared following the conventional practice by subjection of sticky rice (15 kg) to soaking with 20 L of tap water for 4 h, steam cooking, and cooling to

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ca. 30 °C. Then, the rice was mixed with red-koji, white-koji, and reverse-osmosis (RO) water to prepare solid-state moromi[17] and deposited in porcelain jars for fermentation. The jars were sealed with high-density plastic film and fermented at ambient temperature (22−27 °C) for 1.5 months. Red-koji wine broth was obtained by subjection of the fermented moromi to press-filtration through a high-density filtration cloth. The pH values and ethanol and sugar concentrations of the wine broth were 4.2−4.5,12−13% (w/v), and 19.1%, respectively. The wine broth was used as the substrate for vinegar fermentation.

Red-koji vinegar fermentation was conducted following the indigenous practices and slightly modified from the previous procedure.[16] The *Acetobacter* starter used in this study was kindly provided through the indigenous practices of a local rice vinegar factory, and the mixed cultures of strains mainly contained *Acetobacter aceti* subsp. *xylinum*, capable of cellulosic gel formation. The floating transparent jelly *Acetobacter* clumps were dispersed by a homogenizer into suspension and used as the seed starter. As a laboratory-scale batch, 500 mL of the red-koji wine broth was diluted with 500 mL of water and deposited into a 2 L glass jar. For fermentation, each jar was inoculated with 5% of the seed starter suspension (50 mL), wrapped with a kitchen towel paper, and sealed with a rubber band. The whole jar was wrapped with heavy duty wrapping paper to eliminate light exposure and then fermented under ambient temperature (22−27 °C). Three jars for each experiment were conducted. During fermentation, aliquots (15 mL) of the fermented broth were withdrawn every 3 days, deposited in a series of 20 mL vials, and stored at −20 °C for later analyses.

After 30 days of fermentation, the broth from each jar was filtered through four-layered cheesecloth and distributed into a glass bottle and capped. The bottles were heated in a water bath at 70 °C for 10 min. After cooling, the bottles were wrapped with a heavy duty wrapping paper to eliminate light exposure and stored under refrigerated temperature (4−5 °C) for analyses in a week.

Stability of Monacolin K and Citrinin during Fermentation. For assessment of stability, the diluted red-wine substrates were introduced with monacolin K (2.0 μg/mL) and citrinin (3.6 μg/mL) (Sigma Chemical Co., St. Louis, MO, USA) and subjected to fermentation for 30 days. For extraction, 3 mL of the red-koji vinegar was deposited into a 20 mL separation funnel, mixed with 10 mL of ethyl acetate (EA), and vigorously shaken for extraction. After collection of the EA layer, the residual vinegar was re-extracted twice with 10 mL of EA. The EA extracts were pooled and evaporated to dryness with a vacuum rotary evaporator, and the dry residue was reconstituted with 300 μL of acetonitrile. After membrane filtration (0.45 μm), the filtrate was subjected to HPLC quantification. HPLC analysis was performed following the United States Pharmacopeia (USP) Lovastatin procedure (USP29-NF24) with minor modification.[18] An HPLC quantification with an ODS column monitored at 238 nm (Hypersil, C18, 250 × 4.6 mm, 5 μm, Thermo Electron Co., Kleinostheim, Germany) was run with a mobile phase of acetonitrile/0.5% phosphoric acid, 65:35; flow rate, 1.0 mL/min; pump, L-7100 (Hitachi Co., Tokyo, Japan); and detector, L-7420 UV−vis (Hitachi Co.). Authentic monacolin K was run concurrently as a reference.

For citrinin determination, the extraction from red-koji vinegar was the same as that described above for monacolin K extraction. The vacuum-dried residue was reconstituted with 3 mL of methanol, membrane filtered (0.45 μm), and subjected to HPLC analysis following the procedure reported by Wang et al.[19] with minor modification. An HPLC analysis was conducted with equipment of an ODS column (HyPurity C18, 250 × 4.6 mm, 5 μm, Thermo Electron Co.) run with a pump (L-7100, Hitachi Co.) and a fluorescence detector (L-7485 FL detector, Hitachi Co.) with excitation λ_{max} at 330 nm and emission λ_{max} at 500 nm. The mobile phase and flow rate were acetonitrile/water/trifluoroacetic acid (550:450:1, v/v/v) and 1.0 mL/min, respectively.

Compositional Analyses during Red-Koji Vinegar Fermentation. The pH values and acidities of the fermented broth were determined simultaneously. For pH value measurement, 30 mL of vinegar was transferred to a 100 mL beaker, and the pH value was determined using a pH meter (MP220, Mettler Toledo GmbH, Greifensee, Switzerland). To determine acidity, 1 mL of vinegar was diluted with 40 mL of deionized water and titrated with 0.05 N NaOH solution to the pH value of 8.6 by using an automatic titrator (Mettler DL12 Titrator Mettler Toledo AG). The acidity was expressed as percentage of acetic acid (%, w/v).

For determination of soluble carbohydrates and glucose contents, the procedure was followed as previously described.[20] Briefly, 20 μL of the vinegar samples (some were appropriately diluted with deionized water) was mixed with 1 mL of a 0.2% anthrone solution (dissolved in 70% H_2SO_4) in a series of glass test tubes and heated in a water bath at 100 °C for 10 min. After cooling in an ice bath for 5 min, their absorbance at 625 nm was measured. An authentic glucose (Sigma Chemical Co.) solution (1.0 mg/mL) was determined concurrently for estimation of the soluble carbohydrate contents.

For determination of the glucose contents, the procedure reported by Saeki[21] was followed with modification: 0.1 mL the fermented broth was diluted with 0.9 mL of 0.06 N NaOH, membrane-filtered (0.45 μm), and subjected to analysis with HPLC (Hamilton RCX-10 column, 250 × 4.6 mm, Hamilton Co., Reno, NV, USA; mobile phase, 0.06 N NaOH; flow rate, 1.0 mL/min; pump, series III digital pump, Lab Alliance, State College, PA, USA; temperature, 40 °C with an Eldex CH-150 column oven, Eldex Laboratories, Napa, CA, USA; detector, refractive index detector, RI 2000, Schambeck SFD GmbH, Germany; and integrator, D-2500 chromato-integrator, Hitachi Co.). Authentic glucose (Sigma Chemical Co.) was used as reference and run concurrently for quantification.

In the analysis of ethanol and organic acid compositions, the procedure reported was followed with modification.[22] One hundred microliters of the fermented broth was diluted with 0.9 mL of 0.0085 N H_2SO_4, deposited in a 2 mL microfuge tube, and capped tightly. After the tube had been heated in a water bath at 70 °C for 5 min and cooled to ambient temperature, the solution was membrane-filtered (0.45 μm) and analyzed with an HPLC (series III digital pump, Lab Alliance; Eldex CH-150 column oven, Eldex Laboratories; refractive index detector, RI 2000, Schambeck SFD GmbH; and D-2500 chromato-integrator, Hitachi Co.) with an ICSep ICE-ION-300 column (Transgenomic, Inc., San Jose, CA, USA) and run at 68 °C with 0.0085 N H_2SO_4 as mobile phase at 0.4 mL/min. Authentic standards of ethanol, fumaric acid, oxalic acid, citric acid, lactic acid, acetic acid, malic acid, tartaric acid, gluconic acid, and succinic acid were run concurrently as references for quantitative and qualitative analyses.

For determination of amino acid compositions in the prepared red-koji wine substrate, the reported procedures were followed with modification.[23] Two milliliters of the broth at 0 days of fermentation was deposited into a microfuge tube and centrifuged (19000*g* for 2 min), and the supernatant was membrane-filtered. From this was withdrawn 1 mL of wine, which was deposited into a round flask. After rotary vacuum evaporation to reach about half the original volume to evaporate ethanol, the remaining solution was diluted with 0.01 M HCl to 5 mL. After membrane filtration (0.2 μm), the filtrate was subjected to amino acid analysis with an amino acid analyzer (L-8500 AAA, Hitachi Co.). For determination of amino acid compositions of the red-koji vinegar during fermentation, 2 mL of the fermented broth was sampled, deposited into a 2 mL Eppendorf tube, heated at 70 °C for 5 min, and centrifuged (19000*g* for 2 min). The supernatant was diluted with 0.02 M HCl to 5 mL, membrane-filtered (0.2 μm), and subjected to amino acid analysis.

Total Phenolics Determination. The reported procedures were followed with modification.[24,25] Briefly, 0.1 mL of the approximately diluted vinegar samples was mixed with 0.5 mL of Folin−Ciocalteu's phenol regent (Sigma Chemical Co.) and incubated at ambient temperature for 3 min. Then, 0.4 mL of 7.5% Na_2CO_3 was added and incubated for an additional 30 min prior to absorbance determination at 750 nm. A series of gallic acid (Sigma Chemical Co.) solutions containing 0, 25, 50, 75, and 100 ppm (μg/mL) were prepared and used as standards for quantification.

Bioactivity Determination. For extraction of bioactive ingredients, 10 mL of the red-koji vinegar was thoroughly and vigorously

mixed with 10 mL of EA. After two-phase partition, the upper EA layer was collected and the remaining vinegar layer was re-extracted twice with EA. Then, the EA extracts were pooled and evaporated to dryness with a rotary vacuum evaporator. The dried residue was reconstituted with 10 mL of deionized water and assigned as EA extract. The remaining water layer was flushed with nitrogen to diminish the residual EA and replenished with deionized water to reach 10 mL and assigned as the water layer.

For determination of AOP and DPPH scavenging activity, the reported procedures were followed.[26] A pro-oxidant solution was prepared by dissolving $FeCl_3$ and ascorbic acid in 50 mM Tris-HCl buffer solution (pH 7.4) to reach 30 and 200 μM, respectively. For preparation of substrate solution, 100 mg of linoleic acid (Sigma Chemical Co.) and 1 g of Tween-20 were dissolved in 20 mL of Tris-HCl buffer solution and ultrasonicated in an ice bath for complete emulsification. The substrate solution was freshly prepared for each batch of determination.

For each determination, 100 μL of sample was mixed with 500 μL of the pro-oxidant solution and 500 μL of substrate solution in a test tube, thoroughly mixed by vortexing, and incubated at 37 °C. For each sample, duplicate reactants in two tubes were prepared. After respectively 5 and 30 min of reaction, 10 μL of 4% BHT (in methanol) was added to cease oxidation, and the tube was immersed in an ice bath. One hundred microliters of reactant was withdrawn and diluted with 2.4 mL of methanol, followed by absorbance determination at 234 nm (U-2001 spectrometer, Hitachi Co.) for AOP estimation based on absorbance increases from 5 to 30 min.

For determination of DPPH scavenging activity, the reported procedures were followed.[27,28] Briefly, 2 mL of the approximately diluted vinegar solution was mixed with 0.1 mL of DPPH solution (in methanol) and incubated at ambient temperature without light exposure for 30 min. Then, the reactants were subjected to absorbance determination at 517 nm.

Xanthine Oxidase Inhibitory Activity Determination. The reported procedure was followed with minor modification.[29] Fifty microliters of the red-koji vinegar sample or saline (as blank) was deposited into a 10 mL test tube. In each tube, 675 μL of $NaHCO_3$ buffer (50 mM, pH 9.4), 100 μL of 1 mM xanthine (Sigma Chemical Co.), 100 μL of 1 mM EDTA, and 75 μL of xanthine oxidase (200 mU/mL) (Sigma Chemical Co.) were added and incubated at ambient temperature. Absorbance at 290 nm of the reactant was determined from 20 s (assigned as 0 min) to an additional 2 min of incubation (U-2001 spectrometer, Hitachi Co.). XO activity was determined on the basis of the production of uric acid in a 2 min reaction and expressed as the percentage inhibition calculated accordingly. Allopurinol (a xanthine oxidase inhibitor) (Sigma Chemical Co.) was used to prepare a series of concentrations and construct a reference curve for estimation of XOI equivalency.

Statistic Analysis. Triplicate experiments were conducted, and means with standard deviations are expressed. Data were statistically analyzed with SigmaPlot software.

RESULTS AND DISCUSSION

Stabilities of Monacolin K and Citrinin during Red-Koji Vinegar Fermentation. Monacolin K is one of the bioactive secondary metabolites produced by *Monascus* koji, also known as lovastatin. It is active in inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-governing enzyme in cholesterol synthesis.[7−9] Inhibition of mevalonic acid formation and subsequent inhibition of cholesterol biosynthesis by monacolin K is widely appreciated. Therefore, lowering human blood cholesterol level by intake of monacolin K from *Monascus*-treated foods is highly expected. In this study, when a high dose of monacolin K (2.0 μg/mL) was artificially introduced to the red-koji wine and subjected to vinegar fermentation, monacolin K contents decreased gradually with time of fermentation (Figure 1). After 30 days of fermentation, 57% of the original level was retained, and this reveals that monacolin K was fairly stable during red-koji fermentation. Monacolin K may be present in inactive lactone form (MKL) or as the active β-hydroxy acid form (MKA). In aqueous solution, equilibrium between MKL and MKA forms is highly pH dependent.[30] MKL is totally converted to MKA in alkaline solutions. In acidic solutions, proportional MKL concentrations increase with decrease of pH values, whereas the interconversion is almost balanced at pH values below 4.5. During red-koji vinegar fermentation, the pH values gradually decreased from 4.2 to 3.8, the acidities increased from 0.37 to 4.5%, and the alcohol concentrations rapidly decreased from 6 to 0% during the first 12 days (Figure 2). As a consequence, the conversion rate of MKL and MKA is minimized and MKL is the predominant form of monacolin K present in the vinegar. As MKL is of poor solubility and easily removed by precipitation from acidic solution, the content of monacolin K determined in the vinegar might be underestimated.

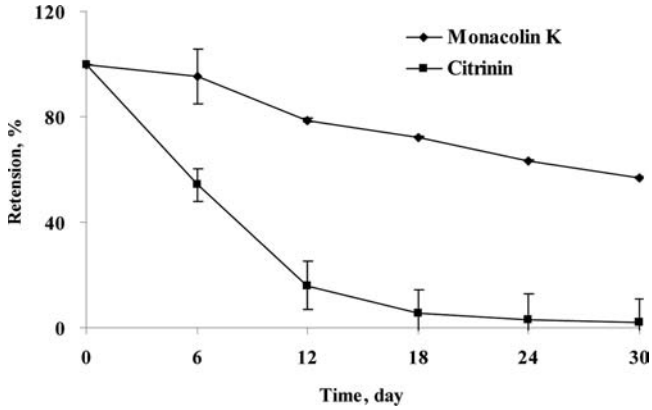

Figure 1. Changes of supplemented monacolin K and citrinin contents during red-koji vinegar fermentation. Each value represents the mean ± SD (n = 3).

On the toxicological aspect, citrinin contamination for the *Monascus*-involved foods is of concern to the public. Citrinin is biosynthesized by *Monascus* or other contaminant molds and identified as a hepatoxin causing kidney or liver toxicity.[1,10] Citrinin content of 0.1−122 mg/kg was found in commercial red-koji fermented rice products.[10,31] According to the Taiwan Food and Drug Administration (TFDA), the maximum allowed levels of citrinin in red fermented rice and red fermented rice-containing product are 5 mg/kg (5 ppm) and 2 mg/kg (2 ppm), respectively.[32] Thus, eliminating its contents below the allowed level is an important issue of food safety regulation. When the authentic citrinin 3.6 μg/mL was artificially introduced to the red-koji wine and subjected to red-koji vinegar fermentation, citrinin concentrations decreased considerably with time of fermentation (Figure 1). After 30 days of fermentation, concentration of the retained citrinin is 0.06 μg/mL (1.9% of the original citrinin level), which is much lower than the maximum allowed level of 2 ppm. This observation indicates that citrinin was unstable during red-koji vinegar fermentation. It has been reported that citrinin produced by *Monascus ruber* is highly degradable in the organic acid accumulated condition.[33] The reduction of citrinin in the fermented broth as affected by change of citrinin solubility due to decrease of ethanol concentration during fermentation is also likely. Nevertheless, citrinin contents in the red-koji wine being

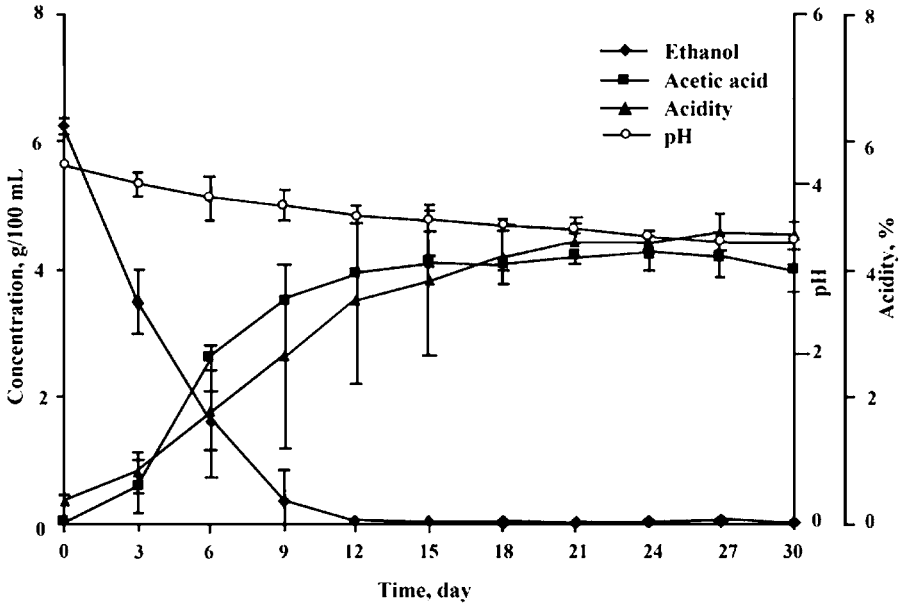

Figure 2. Changes of pH, acidity, and ethanol and acetic acid contents of red-koji vinegar during fermentation. Each value represents the mean ± SD (n = 3).

largely reduced by red-koji vinegar fermentation is of merit from the viewpoint of food safety consideration.

Compositional Changes during Fermentation. Changes of pH value and acidity of the red-koji vinegar broth during fermentation are shown in Figure 2. The broth pH values decreased from 4.2 to 3.3, and its acidities increased from 0.37 to 4.5% (w/v expressed as acetic acid) after 30 days of fermentation. In the initial 12 days of fermentation, ethanol concentration decreased from 6.2% to a nondetectable level. During fermentation, acetic acid contents increased rapidly from 0.02 to 4.26%.

For the other organic acids (Figure 3), as referenced to the authentic standards detected by HPLC, gluconic and malic acids were not well-resolved and the contents of these two acids were very low and ignored in the detected samples. As observed, it is of note that lactic acid contents decreased from 0.31% (w/v) to nearly nondetectable after 21 days of fermentation. This was in agreement with the results of Morales et al., who reported that lactic acid could be consumed by *Acetobacter* and *Gluconobacter* starters.[22] During fermentation, citric acid, succinic acid, and tartaric acid contents increased slightly from 0 to 0.02%, from 0.07 to 0.11%, and from 0.002 to 0.005%, respectively. Fumaric acid contents changed in a limited range from 0.36 to 0.35%. As generalized, ethanol was the major and lactic acid the minor sources of substrate used for production of acetic acid during red-koji vinegar fermentation.

After 30 days of fermentation, total soluble carbohydrate and glucose contents decreased from 9.53 to 8.83% and from 7.8 to 7.0%, respectively (Figure 4). Apparently, only a small amount of soluble carbohydrates or glucose was consumed during vinegar fermentation. In comparison to rapid consumption of ethanol during fermentation (Figure 2), it seems that ethanol was the most favorable choice rather than glucose as carbon source by the starters used for acetic acid production. This might be related to the nature of *Acetobacter* starter, which is capable of oxidizing ethanol to acetic acid and further utilizing acetic acid and lactic acid as carbon source and eventually releasing CO_2 and H_2O.[34] An edible cellulosic gel was

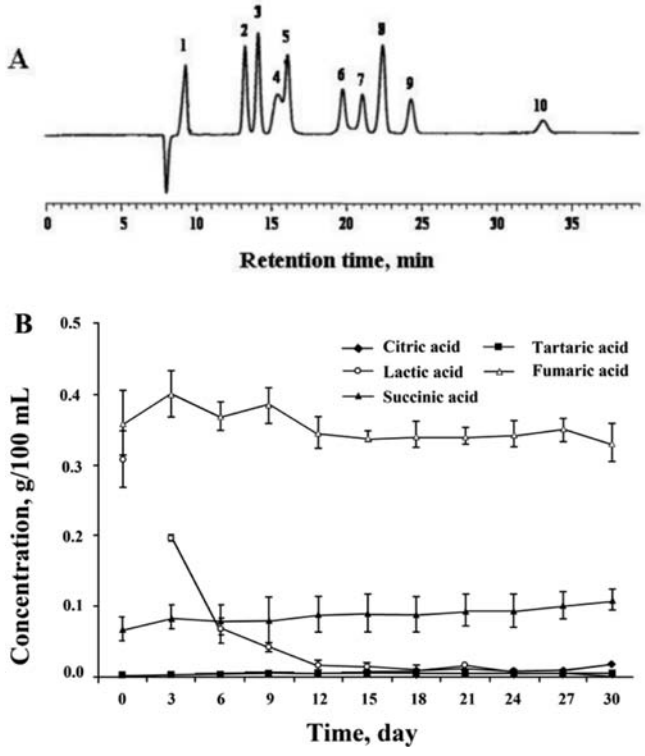

Figure 3. (A) HPLC chromatograms of various organic acids. Peaks: 1, oxalic acid; 2, citric acid; 3, tartaric acid; 4, gluconic acid; 5, malic acid; 6, succinic acid; 7, lactic acid; 8, fumaric acid; 9, acetic acid; 10, ethanol. (B) Changes of citric acid, lactic acid, succinic acid, tartaric acid, and fumaric acid contents of red-koji vinegar during fermentation. Each value represents the mean ± SD (n = 3).

commonly biosynthesized by *A. aceti* subsp. *xylinum*, mainly through the pentose phosphate cycle.[35,36] The starter also produces ethanol, acetic acid, and pyruvic acid through the citric acid cycle.[37] Under specified conditions, the starter may biosynthesize glucose by gluconeogenesis and further biosynthesize the cellulosic gel.[38] The observed cellulosic gel

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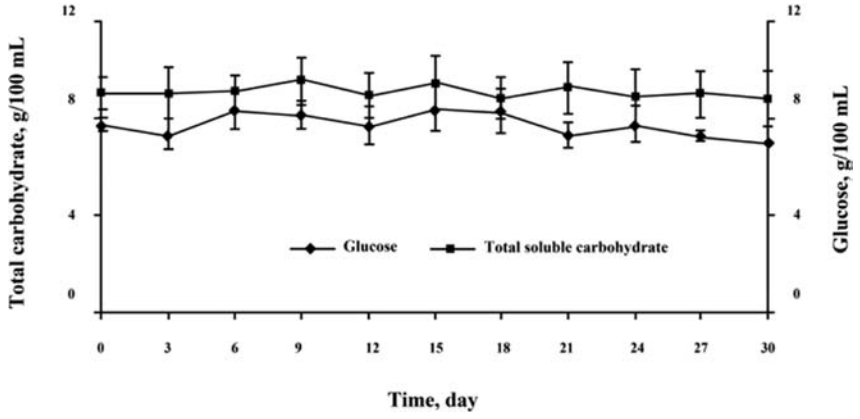

Figure 4. Changes of total soluble carbohydrate and glucose contents of red-koji vinegar during fermentation. Each value represents the mean ± SD (n = 3).

Table 1. Changes of Free Amino Acid Content during Fermentation of Red-Koji Vinegar

	fermentation and amino acid concentration[a] (mg/100 mL)			
amino acid	0 days	9 days	18 days	30 days
aspartic acid	0.392 ± 0.093			
threonine	0.110 ± 0.019	0.113 ± 0.015	0.136 ± 0.027	0.192 ± 0.033
serine	0.202 ± 0.042	0.181 ± 0.035	0.193 ± 0.055	0.229 ± 0.074
glutamic acid	0.381 ± 0.053	0.251 ± 0.051	0.206 ± 0.107	0.180 ± 0.164
glycine	0.221 ± 0.037	0.225 ± 0.034	0.242 ± 0.074	0.272 ± 0.105
alanine	0.397 ± 0.054	0.605 ± 0.174	0.445 ± 0.339	0.359 ± 0.483
cystine	0.052 ± 0.006	0.061 ± 0.004	0.067 ± 0.008	0.085 ± 0.011
valine	0.197 ± 0.038	0.220 ± 0.025	0.260 ± 0.045	0.356 ± 0.049
methionine	0.089 ± 0.023	0.075 ± 0.023	0.071 ± 0.025	0.096 ± 0.049
isoleucine	0.138 ± 0.025	0.148 ± 0.016	0.166 ± 0.029	0.235 ± 0.032
leucine	0.435 ± 0.075	0.419 ± 0.055	0.485 ± 0.092	0.664 ± 0.099
tyrosine	0.422 ± 0.033	0.405 ± 0.037	0.494 ± 0.051	0.754 ± 0.051
phenylalanine	0.431 ± 0.051	0.432 ± 0.039	0.503 ± 0.084	0.738 ± 0.099
lysine	0.289 ± 0.046	0.312 ± 0.036	0.378 ± 0.059	0.544 ± 0.065
histidine	0.127 ± 0.012	0.129 ± 0.013	0.158 ± 0.021	0.233 ± 0.025
arginine	0.702 ± 0.085	0.724 ± 0.085	0.888 ± 0.121	1.302 ± 0.124
proline	0.244 ± 0.041	0.059 ± 0.049	0.027 ± 0.033	0.027 ± 0.047
total	4.830 ± 0.687	4.360 ± 0.616	4.718 ± 1.014	6.267 ± 1.126

[a]Mean of determinations with standard deviation (n = 3).

formation, consumption of lactic acid, and limited change of glucose during red-koji vinegar fermentation supported the identification of the starter used in this study as *A. aceti* or that it has played a vital role in the mixed cultures applied for red-koji vinegar fermentation.

When the red-koji vinegar broths were subjected to amino acid analysis (Table 1), total free amino acid contents increased from 4.83 to 6.27 mg/100 mL. During fermentation, a similar trend of increases was observed for the amino acids, including threonine, serine, glycine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. Proline and glutamic acid contents decreased during vinegar fermentation. Alanine contents increased in the initial 9 days and gradually decreased to the initial concentration. Aspartic acid was detected on day 0 and was nondetectable afterward. Apparently, some free amino acids were dynamically altering with time of fermentation. As quantitatively compared, arginine was the highest amino acid component for the fermented vinegar (1.30 mg/100 mL) and leucine, tyrosine, and phenylalanine were also regarded as major amino acids (>0.6 mg/100 mL).

Contents of Total Phenolic Compounds and Antioxidative Activities. After fermentation for 0, 9, 18, and 30 days, total phenolic compound contents of the fermented broth were 55, 63, 69, and 87 ppm (μg/mL, expressed as gallic acid), respectively (Figure 5A). It is apparent that major parts of the total phenolic compounds were originated from the red-koji wine used as substrate for vinegar fermentation. As reported, when the cooked rice was inoculated and incubated with *Monascus* spp., total phenolic compound contents of the incubated rice were higher than those of uninoculated rice.[39,40] When the fermented vinegar was subjected to EA extraction and quantification of the total phenolic compound contents in EA and aqueous layers, the phenolic contents were 113 and 16 ppm (Figure 5B), respectively. As observed in this study, it is of merit to observe that total phenolic compound contents increased gradually with time of vinegar fermentation.

When the red-koji wine, red-koji vinegar, EA extract, and aqueous fraction of the red-koji vinegar were subjected to AOP

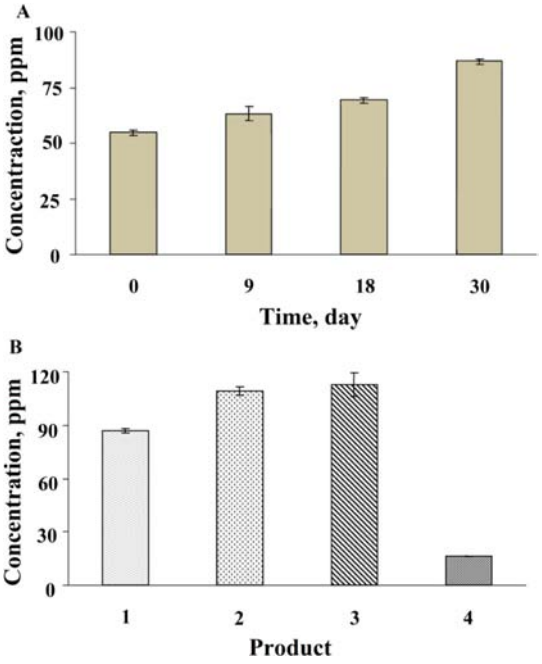

Figure 5. (A) Changes of total phenolics contents of red-koji vinegar during fermentation. (B) Total phenolic compound contents of red-koji vinegar and its related products. Products: 1, red-koji vinegar; 2, red-koji wine; 3, water layer of red-koji vinegar after ethyl acetate extraction; 4, ethyl acetate extract of red-koji vinegar. Each value represents the mean ± SD (n = 3).

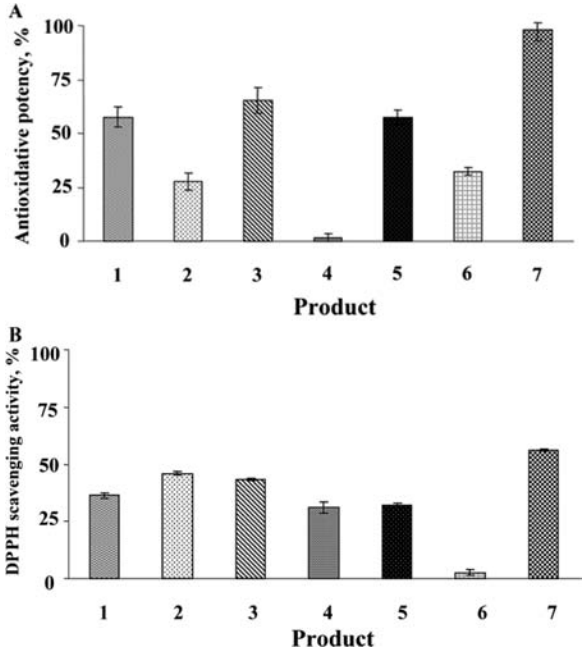

Figure 6. (A) Antioxidative potency (AOP) of various red-koji vinegar solutions and butylated hydroxytoluene (BHT 250 μg/mL) as determined by retardation of linoleic acid oxidation. (B) DPPH scavenging activities of various red-koji vinegar related products and butylated hydroxytoluene (BHT 10 μg/mL). Products: 1, red-koji vinegar; 2, red-koji wine; 3, water layer after ethyl acetate extraction; 4, ethyl acetate extract of red-koji vinegar; 5, acetic acid (4.5%); 6, ethanol (12%); 7, butylated hydroxytoluene (BHT) (10 μg/mL). Each value represents the mean ± SD (n = 3).

determination, AOP was 57.7% for the red-koji vinegar, 27.5% for the red-koji wine, 1.7% for the EA extract, and 65.4% for the aqueous fraction of the red-koji vinegar (Figure 6A). As a reference for comparison, AOPs for 4.5% glacial acetic acid and 12% ethanol were 57.3 and 32.4%, respectively. In comparison, 12% ethanol exhibited about the same AOP activity as did red-koji wine. In addition, 4.5% acetic acid also exhibited about the same activity as did red-koji vinegar. However, after EA extraction, the AOP of the aqueous layer was higher than that of red-koji vinegar (its acetic acid content was not changed after extraction). Some antioxidants such as aqueous soluble phenolic compounds (Figure 5) in addition to ethanol and acetic acid may exist in the fermented red-koji vinegar.

As shown in Figure 6B, the DPPH scavenging activities of red-koji vinegar and red-koji wine were 36.4 and 46.0%, respectively. As compared, DPPH values for 4.5% acetic acid and 12% ethanol were 32.1 and 2.5%, respectively. When the red-koji vinegar was subjected to EA extraction, DPPH values for the aqueous layer and EA extracts were 43.3 and 31.1%, respectively. It is of interest that acetic acid has exhibited significant DPPH scavenging activity. During fermentation of the red-koji vinegar, red-koji wine was 2-fold diluted with water prior to inoculation of starter. Thus, when a comparison was made on the same substrate basis, DPPH activity of the red-koji vinegar increased after vinegar fermentation. This was in agreement with Shimoji et al.,[14] who have identified DPPH radical scavenging compounds in Japanese unpolished rice vinegar, and with Xu et al.,[41] who observed that vinegar melanoidins exhibit potent DPPH scavenging activities. In a recent paper, the antioxidant activity of vinegar produced from distilled residues of the Japanese liquor Shochu featured prevention of oxidative injury and cancer.[13]

Xanthine Oxidase Inhibitory Activities. XO is the enzyme that catalyzes the conversion of hypoxanthine into xanthine and the oxidation of xanthine to form uric acid. Uric acid is normally excreted in urine.[42] High levels of blood uric acid leading to the accumulation of urate in some tissues, in particular, the joints, causes recurrent acute arthritis known as gout. Nowadays, natural bioactive compounds bearing effective prevention of gout formation are highly expected. Allopurinol, a structural analogue of hypoxanthine, is a commonly prescribed drug used for the relief of gout pain. As shown in Figure 7, red-koji wine exhibited XOI activity when the red-koji wine substrate was subjected to vinegar fermentation for 0, 12, 21, and 30 days; the XOI activities were 36.7, 38.6, 39.4, and 55.9% and equivalent to 9.2, 9.7, 9.9, and 14.0 μg/mL allopurinol, correspondingly. As preliminarily observed, 4.5% acetic acid

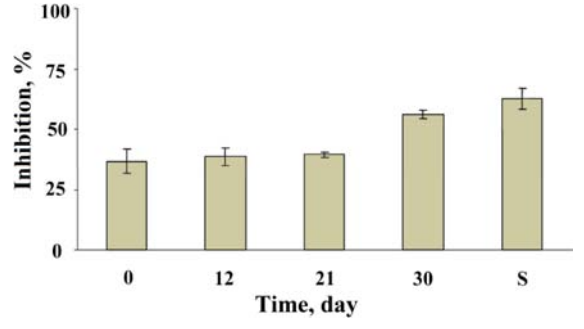

Figure 7. Changes of xanthine oxidase inhibitory activity of red-koji vinegar during fermentation. S, 15 μg/mL allopurinol. Each value represents the mean ± SD (n = 3).

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(about the level of red-koji vinegar) also exhibited 37% activity, which was equivalent to 9 μg/mL of allopurinol. However, when the red-koji vinegar and 4.5% acetic acid were neutralized with 10 N NaOH to pH 7.0 and supplemented with water to the same volume prior to XOI activity determination, the neutralized 4.5% glacial acetic acid lost XOI activity, but the neutralized red-koji vinegar was 32.9% higher than that of the un-neutralized (supplemented with water) red-koji vinegar (data not shown). Obviously, XOI substances were present in both the red-koji wine and vinegar (in addition to acetic acid). The observed XOI increase of the neutralized red-koji vinegar after diminishing acetic acid contribution was supportive of the possible generation of XOI compounds during vinegar fermentation. On the basis of the fact that the additive effect among the original wine XOI, acetic acid, and new generated XOI compounds during red-koji vinegar fermentation was not clearly observed (Figure 7), the original wine XOI compounds might be inactivated during vinegar fermentation, and acetic acid might interact with the newly created XOI compounds. As reported recently, 5-hydroxymethyl-2-furfural (5-HMF) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) have been demonstrated as potent XOI compounds in the red-koji fermented vinegars.[16] Nevertheless, further property characterization and identification of other XOI active compounds in the red-koji wines as well as vinegars would be worthwhile.

As observed, during red-koji vinegar fermentation with *Acetobacter* starter, extensive compositional changes have been detected. Ethanol and lactic acid contents were the major carbon sources rather than soluble carbohydrates or glucose used for acetic acid production. As resulted from fermentation, total phenolic contents, AOP, DPPH scavenging, and XOI inhibitory activities of the red-koji vinegars increased, conferring the vinegar product with nutraceutical merits. When monacolin K (2.0 μg/mL) and citrinin (3.6 μg/mL) were artificially introduced into red-koji wines prior to red vinegar fermentation for 30 days, 43.0 and 98.1% of the original levels were respectively decreased. The likelihood of eliminating citrinin by vinegar fermentation is of merit from the viewpoint of food safety.

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Notes

The authors declare no competing financial interest.

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